Journal of the Korean Magnetic Resonance Society 1997, *1*, 31-44

Binding Aspect of Cyclic AMP Receptor Protein to Symmetrically Synthetic 22-, 28- and 30-Base-Pair *lac* Promoters

Sang-Ho Park, Tae-Woo Lee, Eun-Suk Hwang, Seung-Ki Lee, Cha-Gyun Shin†, and Bong-Jin Lee*

Department of Pharmacy, Seoul National University, Seoul 151-742, Korea
†Department of Biotechnology, Chung-Ang University, An-Sung 456-756, Korea
Received March 21, 1997

Abstract: The effect of the binding of CRP to the symmetrically synthetic 22, 28, and 30 bp *lac* promoter was investigated by ^{1}H NMR. The binding of cAMP*CRP to the 22 bp DNA did not bring about any changes in the chemical shift values, but did cause selective line broadening of imino proton resonances of specific base pairs. However, The binding of cAMP*CRP to the 28 and 30 bp DNA brought about large changes on the imino proton resonances that seems to be induced by DNA bending. We studied also the role of cAMP as an activator of DNA/CRP complex formation by gel mobility shift assay. Gel mobility shift assay revealed that the cAMP*CRP complex was not able to bind to the 22 bp DNA fragment, but was able to bind to the 28 bp DNA fragment of *lac* promoter region.

INTRODUCTION

CRP, also known as catabolite gene activator protien (CAP), regulates the transcription of several catabolite-sensitive genes in *Escherichia coli*. CRP itself interacts weakly and nonspecifically with DNA. However, its complex with cAMP recognizes a specific sequence of DNA and binds to the DNA strongly, suggesting a conformational changes in CRP upon association with cAMP.[1,2] From our NMR studies, it has been suggested that the contraction of CRP is induced by cAMP binding and the

conformational transition mostly occurs when one cAMP molecule binds to one of the dimer subunits .[3,4]

A large number of oligonucleotides of CRP binding sites have been sequenced and a consensus sequence has been proposed.[5] These sites are 22 bp in length and contain one 5'(TGTGA)3' motif and a second sequence, symmetrically related to the first motif, which exhibits various degrees of symmetry. The completely symmetrical site is known to bind to CRP better than a natural site, such as the *lac* promoter site.[6]

In this work, we studied the binding aspect of CRP to 22, 28 and 30 bp DNA to discuss the degree of DNA bending. We also investigated the role of cAMP as an activator of CRP*DNA complex formation. By using gel mobility shift assays, We delineated the mechanism by which cAMP activates CRP to bind to the 22 bp and 28 bp symmetrical DNA fragments containing a TGTGA motif.

MATERIALS AND METHODS

Purification of CRP

CRP was purified from a plasmid-harboring E. *coli* K12 pp47.[7] CRP was purified by cation-exchange chromatography and adsorption chromatography methods. The Bio-rex 70 resin and the hydroxyapatite (HTP) gel were equilibrated with 50 mM potassium phosphate (pH 7.0) and 0.1 mM EDTA (column buffer). After the sample was loaded, the column was washed throughly with column buffer. Bound CRP was eluted with a potassium chloride salt gradient (Bio-rex 70, 0.2-0.8 M KCl; HTP, 0.4-1.0 M KCl) in column buffer. Purified samples were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and were concentrated by ultrafiltration (Centricon, Amicon). The concentration of CRP was determined spectrophotometrically with $\varepsilon_{280} = 4.1 \times 10 M^{-1} cm^{-1}$ for the dimer.

Synthetic DNA Fragments

Three oligonucleotides were prepared by phosphoamidite synthesis on a 10 μ mol scale, using a model 381A DNA synthesizer (Applied Biosystems Co.) :

22 bp *lac* promoter oliogonucleotide, 5'-TAATGTGAGTTAGCCTCACTCAT-3'

22 bp symmetrical oligonucleotide, 5'-TAATGTGAGTTAACTCACATTA-3'

28 bp symmetrical oligonucleotide,

5'-AATTAATGTGAGTTAACTCACATTAATT-3'

30 bp symmetrical oligonucleotide,

5'-TAATTAATGTGAGTTAACTCACATTAATTA-3'

These oligonucleotides were annealed by heating at 90 ℃ for 3 min, followed by slow-cooling to room temperature. Double-stranded oligonucleotides were purified by passage through pyridine Dowex, sodium Dowex, Chelex (BIO-RAD), and Sephadex G-25 columns (Pharmacia). End-labeling with [γ P] ATP using T_4 polynucleotide kinase was performed according to the manufacturer's instructions (New England BioLabs, Beverly, MA).

NMR Measurements

^{1}H-NMR spectra were recorded on JEOL GSX 400 and Bruker AMX 500 NMR spectrometers. Chemical shift values are quoted in ppm downfield from the methyl group resonance of 2,2-dimethyl-2-silapentane-5-sulfonate. The NMR spectra were recorded at 30℃ and the pH of the samples was 6.7. Spectra in H_2O were recorded by means of the 1-1 echo water suppression pulse technique.

Gel Mobility Shift Assay

In general, 6.5, 13, and 26 ng of CRP with/without cAMP was incubated with 2

ng of ^{32}P-labeled DNA fragment at 25℃ for 1 hour in a reaction buffer consisting of 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol, and 50 mM NaCl in a final volume of 25 μl. The mixtures were analyzed by electrophoresis in a 6% nondenaturing polyacrylamide gel. The buffer was 0.5×TBE buffer (45 mM Tris-HCl, 44 mM boric acid, and 10 mM EDTA). The gel was run at 180 V for 1hour at 4 ℃, and was fixed in a mixture of 5% acetic acid and 5% methanol for 30 minutes. The gel was then dried and exposed to film.

RESULTS

Assignment of the oligonucleotide imino proton resonances

The imino proton resonances of the oligonucleotides were assigned from the NOE effects on the imino proton of their neighboring base pairs in a series of one-dimensional NOE experiments (Fig. 1). This assignment was ascertained by temperature-dependent broadening of the imino proton resonance; with increased temperature, broadening of the signal occurred in a sequential manner form the termini. The 28 and 30 bp DNA contain the same sequence as the 22 bp symmetrical DNA with an additional AAT and TAAT sequence at both terminals, respectively. Thus, the assignment of the GC imino proton region is unaffected, but it is hard to assign the additional AT base pair due to the severe overlap of the AT imino proton resonance region around 13.4 ppm (Fig. 2, Fig. 3).

*Effects of cAMP*CRP on the imino proton resonances of the symmetrical 22, 28 and 30 bp lac promoter fragment*

When cAMP*CRP was added to the 22 bp DNA, specific line broadening of the imino proton resonances was observed, no changes in the chemical shift values was

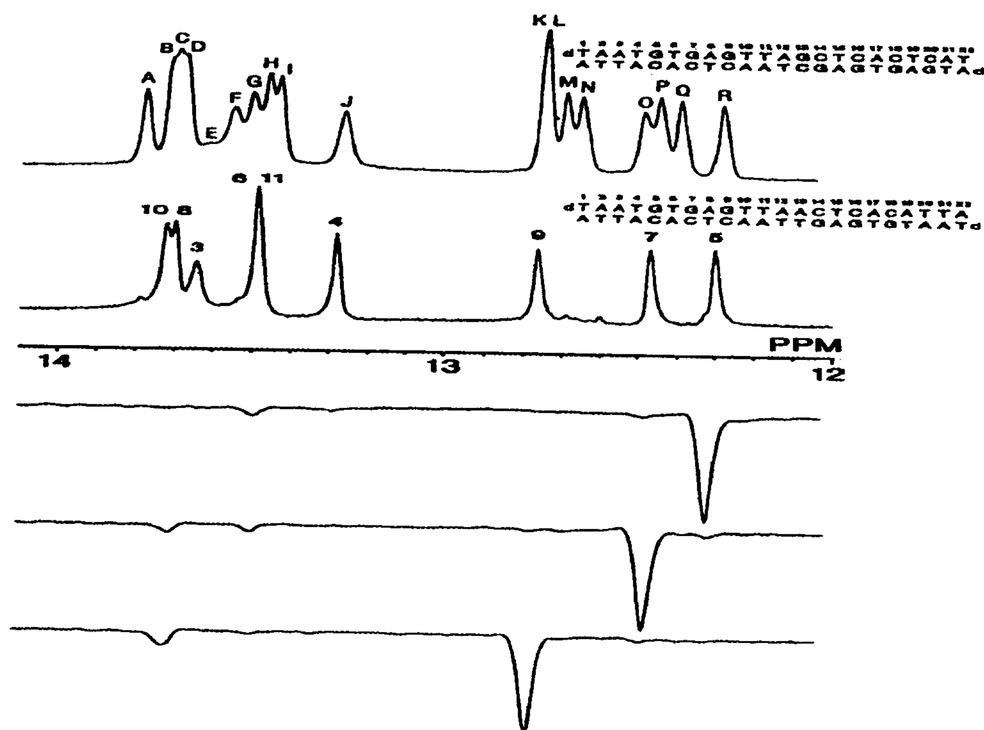

Fig. 1. The reference and NOE difference spectra (off-resonance minus on-resonance) of the 22 bp oligonucleotides. The top spectra are reference spectra of the 22 bp lac promoter oligonuleotide and the 22 bp symmetrical one, respectively. The imino protons of the GC base pairs of the symmetrical 22 bp DNA were irradiated sequentially. The imino protons of AT base pairs also were irradiated sequentially (data not shown). The concentration of the DNA was 1 mM. The buffer was 50 mM potassium phosphate and 0.2 M KCl, pH 6.7.

detected. Specifically, resonances of two base pairs (TA 4, GC 5) and the resonance at 13.5 ppm that contains the resonances of TA 6 and TA 11 were broadened as a result of the complex formation with cAMP*CRP (Fig. 2). When cAMP*CRP was added to the 28 bp DNA, however, large signal changes were observed. The resonances of the base pairs (TA 4, GC 5, GC 7, and GC 9) and the resonance of down-field region around 13.45 ppm were weakened. In addition, new peaks

appeared at the shoulders of peaks at 12.56 and 12.55 ppm, as indicated by the arrows (Fig. 3). This results show that bound cAMP*CRP induces a significant structural change in the DNA.

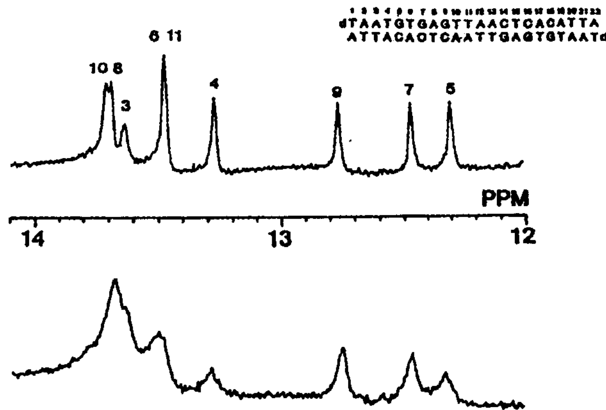

Fig. 2. Effects of the binding of cAMP*CRP on the imino proton resonances of 22 bp DNA. cAMP*CRP was titrated to DNA with ratio of 1.0. The molar ratio of cAMP to CRP was 5. The concentration of 22 bp DNA was 0.3 mM. The spectra were recorded in 50 mM potassium phosphate, 0.2 M KCl, 90% H2O, 10% D2O, pH 6.7, 30 ℃.

From the pattern of the resonance changes, it seems likely that cAMP*CRP induced DNA bending. When cAMP*CRP was added to the 30 bp DNA, the overall changes of resonances were same with those of 28 bp DNA (Fig. 4). Therefore, it can be thought that the bending degree of 30 bp DNA is same with that of 28 bp DNA

Gel Mobility Shift Assays

Gel mobility shift assays are a facile method to determine the specificity of protein binding to DNA, and to provide a means of quantitating DNA/protein complex formation.[8] We used a gel mobility shift assay to study how cAMP activates

CRP to bind to 22 bp and 28 bp symmetrical DNA fragments containing a TGTGA motif, which were derived from the lac promoter. To determine the lowest amount of CRP that would provide an optimal level of complex, we used a fix concentrated CRP with cAMP. Fig. 5-a indicates that 26 ng of CRP was the amount ed amount of labeled 22 bp and 28 bp DNA fragments (2 nM) and aliquots of highly concentrated CRP with cAMP.

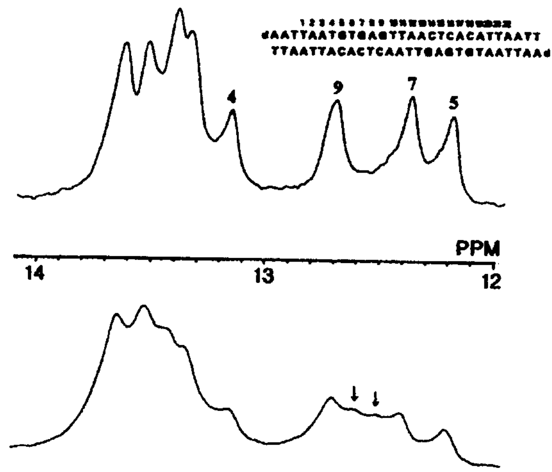

Fig. 3. Effects of by the binding of cAMP*CRPon the imino proton resonances of 28 bp DNA. cAMP*CRP was titrated to DNA with ratio of 1.0. The molar ratio of cAMP to CRP was 5. The concentration of 28 bp DNA was 0.3 mM. The experimental condition was same with Fig. 2.

Fig. 5-a indicates that 26 ng of CRP was the amount that allowed the formation of the greatest amount of complex; below 26 ng, complex formation was reduced. The temperatures for the incubation and the electrophoresis conditions were optimized to enhance the stability and activity of DNA/protein binding. We also compared the binding affinity of CRP to the 22 bp DNA with that of the 28 bp DNA with/without cAMP, respectively. Fig. 5-b shows that CRP did not bind to the 22 bp DNA, but would bind to the 28 bp DNA only when CRP was activated by cAMP binding.

DISCUSSION

CRP possesses a helix-turn-helix motif, which has been shown to be present in many bacterial DNA-binding pro teins. It has been proposed that the helix-turn-helix motif of CRP interact with DNA.[9] In addition, C-terminal region of CRP has been proposed to participate in DNA binding and to be important in DNA bending.[10]

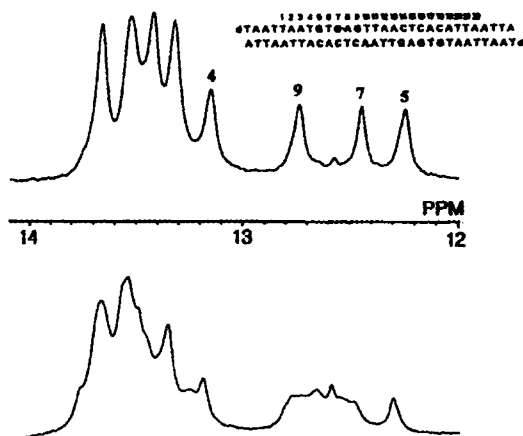

Fig. 4. Effects of the binding of cAMP*CRP on the imino proton resonances of 30 bp DNA. cAMP*CRP was titrated to DNA with ratio of 1.0. The molar ratio of cAMP to CRP was 5. The concentration of 30 bp DNA was 0.3 mM. The experimental condition was same with Fig. 2.

The ^{1}H NMR resonances of the DNA imino protons can be conveniently used for the detection of local changes in the helix conformation induced by the binding of a protein, as these resonances do not generally overlap with protein signals. Being located in the interior of the helix, the imino protons provide a sensitive probe for changes in the DNA helix conformation. The binding of cAMP*CRP to 22 bp DNA induces a no changes in the chemical shift values of the imino protons. From gel

electrophoresis analyses, CRP has been shown to induce a bending in its binding site at the lac promoter.[6] Therefore, no change in the chemical shift values is incompatible with the model of DNA bending. However, the binding of cAMP*CRP
to 28 and 30 bp DNA induces changes in the chemical shift values of the imino proton resonances and induces a specific line broadening in two imino proton resinances (TA 4, GC 5). Therefore the binding of cAMP*CRP to the 28 and 30 bp

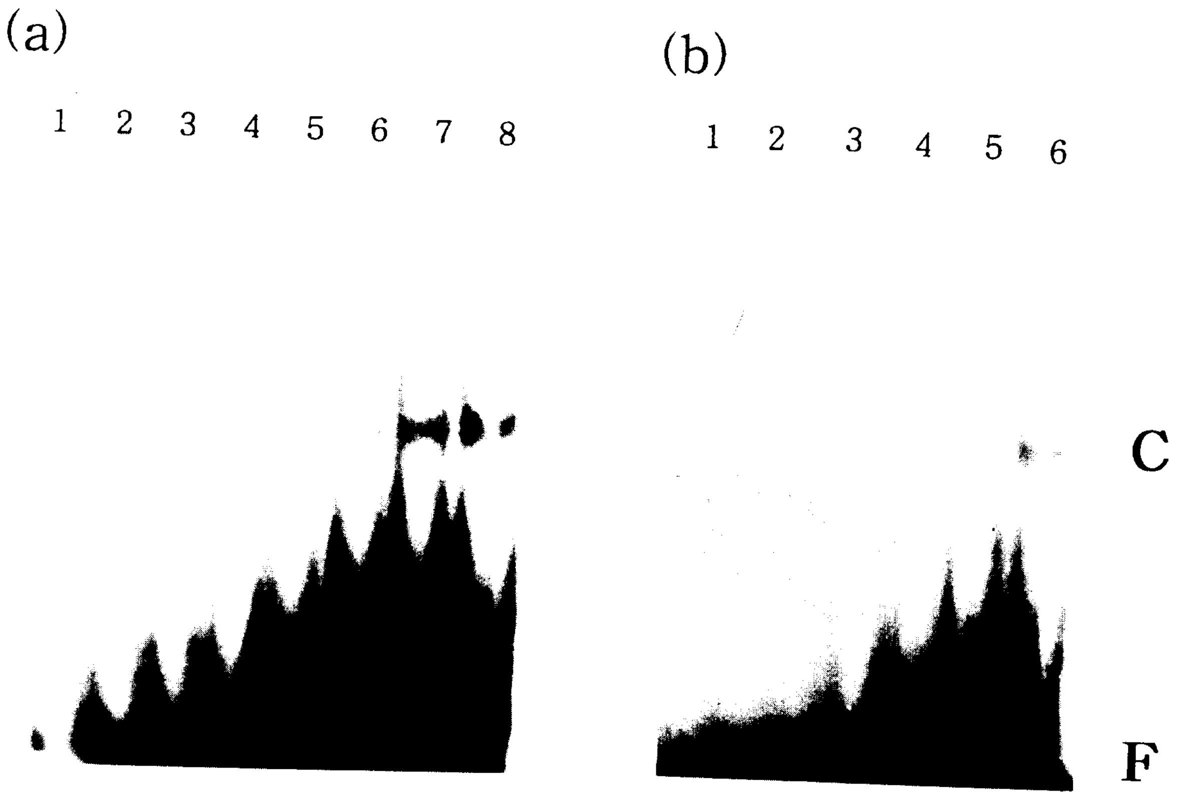

Fig. 5. (a) Determination of the optimal binding activity of cAMP*CRP (5:1) on the 22 bp and 28 bp DNA. 2 ng 32P-labeled DNA fragment was incubated with varying levels of cAMP*CRP, followed by electrophoresis through a 4% native polyacrylamide gel. The results are visualized by autoradiography. Lane 1 and lane 5 contain labeled 22 bp and 28 bp DNA fragments without cAMP*CRP addition as a DNA control, respectively. Lanes 2～4 and lanes 6～8 are the labeled 22 bp and 28 bp DNA fragment, respectively, incubated with cAMP*CRP at concenrations of 6.5, 13, and 26 ng. (b) Determination of the binding activity of CRP on the 22 bp and 28 bp DNAs with and without cAMP. Lane 1 and lane 4 contain

labeled 22 bp and 28 bp DNA as a control, respectively. Lane 2 is the labeled 22 bp DNA fragment incubated with CRP at a concentration of 26 ng. Lane 3 is the labeled 22 bp DNA fragment incubated with the cAMP*CRP (5:1) complex at same concentration of CRP. Lane 5 and lane 6 are the labeled 28 bp DNA fragment incubated with CRP and the cAMP*CRP complex at a 26 ng concentration of CRP. The letter C indicates the cAMP*CRP*DNA complex (upper bands), and the letter F indicates the free 22 bp and 28 bp DNAs (bottom bands).

DNA seems to induce DNA bending. It is likely that CRP requires DNA longer than a 22 bp to bend it. Since the aspect of spectral change of 30 bp DNA was same substantially with that of 28 bp DNA, it can be easily imagined that the bending degree of 30 bp DNA is same with that of 28 bp DNA. In addition to the 22 bp consensus sequence, the 28 and 30 bp DNA contain distal binding domains that contribute to the full protein binding affinity and seem to be important for DNA bending. The distal binding domains may wrap around the CRP, stabilizing the binding and leading to a bending in the DNA. Interactions with these distal binding domains are thought to be electrostatic and non-specific. Judging from our experiments, it could be suggested that the helix-turn-helix motif of CRP interacts with 5-bp consensus sequence, 5'TGTGA3', of DNA, and C-terminal region of CRP interacts with terminal base pairs of 28 bp and 30 bp DNA. On the assumption that the length of DNA sequence is longer than 28 base pair, it could be infered that the bending aspect of cAMP*CRP to DNA would be same. However, binding aspect of cAMP*CRP to longer DNAs than 30 bp have to be studied, to gain more accurate information about the bending aspect of DNA.

NMR data demonstrated that cAMP*CRP bound to symmetrical 22 bp and 28 bp DNA fragments containing a TGTGA motif, derived from the *lac* promoter and 28 bp DNA was bent by the binding of cAMP*CRP. However, gel mobility shift assay showed that CRP did not bind to the 22 bp DNA, but would bind to the 28 bp DNA only when cAMP was added to CRP. The difference between the NMR results and gel

mobility shift assay results can be explained as followings: Since a very concentrated sample was used in the NMR studies, compared to that in the gel mobility shift assays, the cAMP*CRP could bind to the 22 bp and 28 bp DNAs in NMR experiments, regardless of their binding affinity. Since the binding constant of the cAMP*CRP to the 22 bp DNA was weaker than that to the 28 bp DNA, the cAMP*CRP could not bind to 22 bp DNA at a very low concentration, as in gel mobility shift experiments.

Acknowledgement

We would like to thank prof. Yoshimasa Kyogoku in Osaka University and prof. Hiroji Aiba in Nagoya University for their generous gift of the CRP gene. This work was supported by the Genetic Engineering Research Program (1995), Ministry of Education, Korea.

REFERENCES

1. B. de Crombrugghe, S. Busby, and H. Buc, *Science* **224**, 831-838 (1984).
2. A. Ullmann, and A. Danchin, *Adv. Cyclic Nucleotide Res.* **15**, 1-53 (1983).
3. B. J. Lee, S. J. Lee, F. Hayashi, H. Aiba, and Y. Kyogoku *J. Biochem.* **107**, 304-309 (1990).
4. B. J. Lee, H. Aiba, and Y. Kyogoku, *Biochemistry* **30**, 9047-9054 (1991).
5. C. Jansen, A. M. Gronenborn, and G. M. Clore, *Biochem. J.* **246**, 227-232 (1987).
6. H. N. Lin-Johnson, M. R. Gartenberg, and D. M. Crothers, *Cell* **47**, 995-1005 (1986).
7. H. Aiba, S. Fujimoto, and N. Ozaki, *Nuceic Acids Res.* **10**, 1345-1361 (1982).
8. K. Jung, H. Jung, and H. R. Kaback, *Biochemistry* **32**, 12273-12278 (1993).

9. S. Busby, *in* "Regulation of Gene Expression: 25 Years on" (I. R. Booth, and C. F. Higgins, Eds.), pp. 51-77, Cambridge University Press, Cambridge, 1986.
10. Z. Yang, S. Bobin, and J. S. Krakow, *Nucleic Acids Res.* **19**, 4253-4257 (1991).

FIGURE LEGNEDS

Fig. 1. The reference and NOE difference spectra (off-resonance minus on-resonance) of the 22 bp oligonucleotides. The top spectra are reference spectra of the 22 bp lac promoter oligonuleotide and the 22 bp symmetrical one, respectively. The imino protons of the GC base pairs of the symmetrical 22 bp DNA were irradiated sequentially. The imino protons of AT base pairs also were irradiated sequentially (data not shown). The concentration of the DNA was 1 mM. The buffer was 50 mM potassium phosphate and 0.2 M KCl, pH 6.7.

Fig. 2. Effects of the binding of cAMP*CRP on the imino proton resonances of 22 bp DNA. cAMP*CRP was titrated to DNA with ratio of 1.0. The molar ratio of cAMP to CRP was 5. The concentration of 22 bp DNA was 0.3 mM. The spectra were recorded in 50 mM potassium phosphate, 0.2 M KCl, 90% H_2O, 10% D_2O, pH 6.7, 30 ℃.

Fig. 3. Effects of by the binding of cAMP*CRPon the imino proton resonances of 28 bp DNA. cAMP*CRP was titrated to DNA with ratio of 1.0. The molar ratio of cAMP to CRP was 5. The concentration of 28 bp DNA was 0.3 mM. The experimental condition was same with Fig. 2.

Fig. 4. Effects of the binding of cAMP*CRP on the imino proton resonances of 30 bp DNA. cAMP*CRP was titrated to DNA with ratio of 1.0. The molar ratio of cAMP to CRP was 5. The concentration of 30 bp DNA was 0.3 mM. The experimental condition was same with Fig. 2.

Fig. 5. (a) Determination of the optimal binding activity of cAMP*CRP (5:1) on the 22 bp and 28 bp DNA. 2 ng P-labeled DNA fragment was incubated with varying levels of cAMP*CRP, followed by electrophoresis through a 4% native polyacrylamide gel. The results are visualized by autoradiography. Lane 1 and lane 5 contain labeled 22 bp and 28 bp DNA fragments without cAMP*CRP addition as a DNA control, respectively. Lanes 2~4 and lanes 6~8 are the labeled 22 bp and 28 bp DNA fragment, respectively, incubated with cAMP*CRP at concenrations of 6.5, 13, and 26 ng. (b) Determination of the binding activity of CRP on the 22 bp and 28 bp DNAs with and without cAMP. Lane 1 and lane 4 contain labeled 22 bp and 28 bp DNA as a control, respectively. Lane 2 is the labeled 22 bp DNA fragment incubated with CRP at a concentration of 26 ng. Lane 3 is the labeled 22 bp DNA fragment incubated with the cAMP*CRP (5:1) complex at same concentration of CRP. Lane 5 and lane 6 are the labeled 28 bp DNA fragment incubated with CRP and the cAMP*CRP complex at a 26 ng concentration of CRP. The letter C indicates the cAMP*CRP*DNA complex (upper bands), and the letter F indicates the free 22 bp and 28 bp DNAs (bottom bands).